Chapter 4.

NO ESCAPE FROM MOTHER'S WILL

Effects of maternal testosterone on offspring reproductive behaviour far into adulthood

Bin-Yan Hsu, Cor Dijkstra, and Ton G.G. Groothuis

Animal Behaviour 117: 135-144 (2016)

Abstract

In many animal species, mothers expose their embryos to maternal hormones. This provides a powerful pathway for affecting offspring phenotype and a potential mechanism for adaptive non-genomic inheritance. This has been studied extensively in birds because the embryo develops outside the mother's body, facilitating experimental studies. Some studies have demonstrated that maternal yolk testosterone (T) can have long-lasting effects into the period after fledging. However, these results are based on offspring in the juvenile stage or their first breeding season only. The lack of data further into adulthood limits a better assessment of the fitness consequences of prenatal T. In a series of previous experiments in the rock pigeons (*Columba livia*), we manipulated yolk T levels using oil-injected eggs as controls. In the present study, we examined the reproductive behaviour of birds from these eggs when they were 1-3 years old and housed together in a large aviary. Our results showed that males from T-injected eggs were less aggressive and females from T-injected eggs laid lighter eggs than controls. Moreover, the experimental birds showed strongly disassortative mating with respect to hormone treatment. There were no indications of treatment effects on the process of sexual differentiation. Our results indicate that the effects of prenatal T do not diminish over age and may last far into adulthood, which should be taken into account in assessing the fitness consequences of maternal hormones. Furthermore, our results may also stimulate new physiological and neurobiological studies on the mechanisms underlying such long-lasting effects of prenatal T exposure.

Keywords:

Aggression, Birds, Courtship behaviour, Long-term effects, Maternal effects, Maternal hormones, Prenatal testosterone exposure, Reproduction

Introduction

In addition to direct genetic effects, the expression of the phenotype can be modified by developmental plasticity, induced by many environmental factors during critical developmental phases, both prenatally and postnatally (Gilbert 2001). A special interesting case is maternal effects in which these non-genetic effects are brought about through the maternal phenotype. Maternal effects can potentially modify the offspring's phenotype to suit the prevailing or expected environment through non-genomic inheritance (Mousseau and Fox 1998). An important pathway for these maternal effects in many species, both viviparous and oviparous, is maternally derived hormones that can reach the embryo either across the placenta or through the egg yolk (Groothuis and Schwabl 2008). In the past few decades, maternal effects have attracted a lot of research interest, especially in birds in which egg hormones can be relatively easily measured and manipulated. There is now convincing evidence that concentrations of hormones in avian egg yolk vary systematically within and between clutches, between females of the same species, in relation to environmental factors, and between species in relation to life history (Groothuis et al. 2005; Gil 2008; von Engelhardt and Groothuis 2011). This, as well as the many studies on the effect of testosterone (T) manipulation in ovo has generated a debate about the adaptive value of hormone mediated maternal effects (Groothuis et al. 2005; W. Müller et al. 2007; Gil 2008; Groothuis and Schwabl 2008; von Engelhardt and Groothuis 2011). The majority of the injection studies have focussed on the short term effects of the manipulation on the chicks, finding an influence on growth rate, early behaviour, and physiology (reviewed in Groothuis et al. 2005; Gil 2008; von Engelhardt and Groothuis 2011). However, early exposure to androgens is well known to have long-lasting effects, either due to the organizational effects on brain and behaviour or by inducing different starting points for further development (Carere and Balthazart 2007; Groothuis and Schwabl 2008). Such long-lasting effects need to be taken into account to evaluate the total fitness effects of exposure to maternal androgens.

To date more than 20 studies have analysed long-term effects of T manipulation in avian eggs with about two third of them finding effects on morphology (Strasser and Schwabl 2004; Eising et al. 2006; Rubolini et al. 2006, 2014; Bonisoli-Alquati et al. 2011), behaviour (Strasser and Schwabl 2004; Eising, et al. 2006; Tschirren et al. 2007; Partecke and Schwabl 2008; Ruuskanen and Laaksonen 2010; Bonisoli-Alquati et al. 2011; Schweitzer et al. 2013), reproduction (Uller et al. 2005; Rubolini et al. 2007; W. Müller et al. 2009), and even fitness estimates (Ruuskanen et al. 2012; Tschirren et al. 2014). However, these results are mixed with enhancing, and suppressive effects whereas several other studies found no effects (e.g. W. Müller and Eens 2009; Vergauwen et al. 2014; for an extensive

review, see von Engelhardt and Groothuis 2011). Explanations for such inconsistency include that some studies have analysed long-lasting effects in juveniles and others in adults, some in artificial captive situations and others in (semi) natural conditions, and that sometimes supra-physiological hormone dosages were applied. Moreover, none of the studies analysed these effects in birds over more than 1 year of age, limiting the interpretation in terms of lifetime fitness consequences. The effects of prenatal androgen exposure might not necessarily last into old ages. W. Müller et al. (2008) found that although exposure to elevated levels of yolk T delayed song development in juvenile male canaries (*Serinus canaria*), there was no significant difference in all measured adult song parameters. The most often cited studies on long term effects of yolk T only presented the effects in the juvenile period (black-headed gulls, *Chroicocephalus ridibundus*; Eising et al. 2006) or at 1 year of age (house sparrows, *Passer domesticus,* Strasser and Schwabl 2004; Partecke and Schwabl 2008). In the latter species the effect of yolk T on mortality appeared to be age specific (Schwabl et al. 2012). Behavioural traits can also change with time by experience and learning, and the effects of early androgen exposure might thus be masked or enhanced over time. In addition, a different start of development induced by exposure to elevated levels of maternal T such as mass, competitiveness, metabolic rate or oxidative stress (for a review see von Engelhardt and Groothuis 2011) might induce different developmental trajectories and different life history decisions (Carere and Balthazart 2007; Groothuis and Schwabl 2008), affecting even the process of ageing, as is suggested by the mortality effects found by Schwabl et al. (2012). These age-dependent effects can have substantial consequences for the fitness effects of maternal hormone deposition and therefore on natural selection.

With the aim of advancing our knowledge about the long-term effects of maternal hormones further into adulthood, we quantified the social and reproductive behaviour of adult rock pigeons (*Columba livia livia*) of both sexes, hatched from eggs injected with either vehicle only or testosterone within the physiological range of this species. In the breeding season in which this study was conducted, the birds were housed together in a very large aviary. The birds were 1-3 years old, allowing us to investigate whether the effect of in ovo T injection waned over time.

Materials and Methods

Study species

In this study we followed 119 adult pigeons that had hatched from either T-injected eggs (T-pigeons hereafter, *N*=63) or oil-injected eggs (C-pigeons, *N*=56) in our previous

experiments. These birds were bred from our pigeon colony which originated from out-bred wild-caught individuals. All pigeons showed the same wild-type "blue bar" plumage pattern (Johnston and Janiga 1995) and white rump feathers on the lower back and they were morphologically distinct from the domestic racing pigeon as they were visibly smaller and with a small operculum.

All experimental and control birds had been housed after fledging and independence in a large outdoor but roofed aviary (45m long × 9.6m wide × 3.75m high) in the animal facility of the Centre for Life Sciences, University of Groningen, which also housed non-experimental pairs. The number of animals housed there varied from 100 to 300. The ground was covered with sand. Food (seed mixture for *Streptopelia* species, KASPER™ 6721 + seed mixture for *Columba* species, KASPER™ 6712 + pellets for pigeons, KASPER™ P40) together with grit and water was provided ad libitum. On 31 March, 2014, we caught all pigeons, checked their leg bands, and measured their body mass.

Over the course of the observation period of this study, in total 163 birds were housed in the aviary. These included 119 birds that were the experimental birds of this study, 34 birds that were involved in another experiment (Hsu, et al., Chapter 5, this thesis), and 10 non-experimental birds that were or became paired with one of the subjects of this study at that time. All these birds were observed but only the 119 experimental birds were included in the data analysis. The age and sex composition of the experimental birds is listed in Table 4.1. The hormone treatment in all previous experiments was the same: injection of either T solution or sesame oil (see *Egg injections*) in the same dosage. In total 78 nestboxes were provided, but remained closed until the middle of the behavioural observation period, allowing us to record the competition for and occupation of nestboxes.

Egg injections

In all previous experiments from 2011 to 2013, we used the same protocol of egg injections (details in M.S. Müller 2013 and Hsu et al. Chapter 3, this thesis). In brief, an egg was placed sideways for a few minutes, allowing the yolk to float up. A 50 µl T solution or sesame oil was then injected using U-100 insulin syringes with 29G needles (BD Micro-Fine™ and Terumo® BS05M2913). After injection, the hole was sealed with a small piece of artificial skin (Hansaplast™). The dose of T solution was 46 ng of T dissolved in 50 µl of sesame oil. The control eggs were injected with 50 µl of sterilized sesame oil only. Rock pigeons typically lay only two eggs as a clutch and the second eggs contain much higher concentrations of yolk T (on average 18.99 pg/mg yolk) than the first eggs (on average 5.18 pg/mg yolk, Hsu et al. 2016). The egg injections were aimed to raise the yolk T levels

Table 4.1 The age and sex composition of the pigeons in this study. All pigeons were derived from a series of experiments on prenatal testosterone exposure in egg yolks. The hormone treatment in all previous experiments was the same: an injection of either 50 µl of testosterone solution (46 ng of testosterone dissolved in 50 µl of sesame oil) or vehicle (50 µl of pure sesame oil) into the egg yolk.

Age	3 years (born in 2011)	2 years (born in 2012)	1 year (born in 2013)	Total
T-female	7	11	9	27
C-female	8	8	7	23
T-male	16	11	9	36
C-male	8	16	9	33
Total	39	46	34	119

T-female/male: females/males hatched from testosterone-injected eggs.
C-female/male: females/males hatched from vehicle-injected (control) eggs.

of the first eggs to the average T levels of the second eggs. For this purpose, only first eggs of a clutch were used in the experiments, and every foster nest received a pair of injected eggs, one with T solution and the other with oil. Since these eggs were all first eggs from their original clutch, no effect of laying order needed to be considered in this study.

Across these experiments, the hatching success was 51-55% (mean±SD: 53.85±1.96%, *N*=3) for T-injected eggs and 46-51% (mean±SD: 48.85±2.83, *N*=3 years) for oil-injected eggs. This difference was not statistically significant (*t* test, $t_{3.562}$=2.510, *P*=0.074). We did not systematically assess the hatching success of un-injected eggs, but it is ca. 70% without predation according to the literature (Hetmański and Barkowska 2007).

Behavioural observations and egg production

From 10 April to 16 May, 2014, at the peak of reproductive behaviour in our colony, we used a group scanning protocol for behavioural observation and recording from an observation corridor next to the aviary. The observers scanned the whole group of pigeons going systematically from one end of the aviary to the other, recording the behaviour of each bird. Two to four of these observation bouts were conducted each day, for 24 days during 6 weeks in total. The direction of each observation scan was randomly determined at the beginning of a day and then alternated, in order to reduce the potential biases due to the location preference of pigeons and the order of the scanning. Every pigeon was identified by a unique combination of three colour bands on their legs. The observers were trained beforehand to become familiar with the protocol and the

behaviour of pigeons. All of the observers were kept blind with respect to knowledge of the individual treatments that the pigeons received.

Courtship, pair-bonding and aggressive behaviour were pre-categorized according to the literature (Johnston and Janiga 1995) and our own experience (Table 4.2). The behaviour of each pigeon at the scanning moment was hand-recorded. By definition, some behaviours could be performed simultaneously by an individual pigeon, for example, tail-dragging and bowing, or tail-dragging and driving. In these cases, both behaviours were recorded. Behaviours were further grouped before data analysis into sex-specific functional categories. For males, tail-dragging, bowing, and the male driving the female were grouped as "Male Courtship" as they are all typical male courtship rituals. For females, tail-dragging and nest-cooing were grouped as "Female courtship"; being driven and being courted were merged as "Being courted." Attacking and fighting by the male were merged into "Aggression". Because females hardly ever engaged in active fighting, but only showed intention pecking at courting males or chasing out nest intruders, female aggression was much less frequent than males and zero-inflated. The analysis of aggression was thus performed separately for each sex. Copulation, courtship feeding and reciprocal preening were usually seen among established pairs, performed by both sexes and grouped as "Pair-bonding".

Each day during the period of 6 weeks, we checked the floor of the aviary and the nestboxes to record egg-laying date and collect freshly-laid eggs. We provided nesting materials and opened the nestboxes on 28 April. Newly laid eggs were collected for another experiment and replaced with dummy eggs. Fresh egg mass was measured with a digital scale (accuracy 0.1 g). In addition to the above-mentioned behavioural observations, we also made additional observations to identify those pigeons that occupied a nestbox or other nesting site as some pigeons preferred to occupy a nest-site on the floor, where pigeons normally reproduce quite well according to our past experience.

Statistical analysis

All statistical analyses were conducted with R 3.0.2 (R core team 2013), mostly using general or generalized linear models (GLM). Yolk hormone treatment (T or C) and age were the independent variables in all models. The quadratic term of age was always included in the beginning, but only retained in the model if it was significant. Sex was included in the models where data were not sex-specific (body mass, nestbox occupation, and body mass), while it was excluded in those models that were analysing sex-specific data (aggression, courtship, female being courted, egg-laying date and egg mass).

Table 4.2 The definitions of pigeon behaviour.

Behaviour	Definition
Courtship-related:	
Tail-dragging (TD)	A pigeon spreads his tail and drags it on the ground.
Bowing (BW)	A pigeon puffs out his neck feathers, lowers his head and turns around in circles, producing a cooing sound (bow-cooing).
Nest-cooing (NC)	A pigeon lowers his head and body, inflates the crop and produces a cooing sound.
Driving (D)	One pigeon runs closely behind another.
Copulation (CP)	A pigeon stands on top of another pigeon, with their cloacae touching.
Courtship feeding (CF)	A pigeon puts her beak inside the beak of the other.
Reciprocal preening (RP)	Two pigeons stand/sit closely and preen each other.
Being courted (C0)	Another pigeon is doing TD, BW towards the targeted pigeon.
Being driven (D0)	Another pigeon is driving the targeted pigeon.
Aggression-related:	
Attacking (A1)	Active agonistic behaviour by a pigeon against another one, i.e. pecking, chasing, beating with wings, or kicking.
Fighting (F)	Two pigeons actively fight with each other.
Other behaviour:	
Nest-building (NB)	A pigeon brings nest materials to its nest-site or tries to make a nest.
Inactive (I)	Standing, sitting, and any other inactive behaviour
Other (O)	Any other behaviour that is not defined above, e.g. self-preening, eating, walking.

These definitions were based on our own experience and those given by Johnston and Janiga (1995). Some categories were further grouped before data analysis: Male Courtship = TD+BW+D; Female Courtship = TD+NC; Pair-bonding = CP+CF+RP; Being courted = C0+D0; Aggression = A1+F.

The total frequencies of behaviour of each category were analysed with GLMs. Except for pair-bonding, female courtship and aggression, all other behavioural data were first square-root-transformed to approximate normality (Shapiro-Wilk tests, all *P* values > 0.29). The data of pair-bonding, female courtship and aggression were zero-inflated, and thus were re-coded as 1/0 (observed or not) and analysed by binomial GLMs. The data of nestbox occupation were also binomial (yes and no for each pigeon) and analysed by a binomial GLM as well. To examine the possibility of non-random mating according to treatment, we performed a Chi-square test on the data for the pairs that consisted of both birds from T- or oil-injected eggs.

In the course of spring the behaviour changed over time, and only analysing the total frequency might not be able to detect the temporal differences between groups. Therefore, we additionally analysed the two main categories of behaviour, courtship and aggression, by analysing the effect of treatment on the change over time by separating the data by weeks, re-coding them as yes/no, using binomial generalised linear mixed models (GLMMs, R package *lme4*, Bates et al. 2014), with pigeon identity as a random factor.

Egg-laying date was analysed sex-specifically. In both male and female models, model residuals were not normally-distributed (Shapiro-Wilk test, $P<0.03$). We therefore rank-transformed the data (model residuals after transformation, Shapiro-Wilk test, $P>0.13$).

Egg mass data were fitted with GLMMs (R package *lme4*, Bates et al., 2014), with the nest identity included as a random factor and laying order of eggs and female body mass (known to correlate strongly with egg mass) as a fixed factor and covariate respectively. In such a model, there is still no reliable way to calculate the degrees of freedom accurately. Therefore we used log-likelihood ratio tests and parametric bootstrapping to get *P* values (for details, see Supplementary materials).

In all models, main effects were always interpreted with the models without any interaction term. For all models, we additionally tested the interaction between hormone and age in order to test whether possible effects of prenatal T diminish gradually with increasing age. For models where sex was included, we also tested the interaction between hormone treatment and sex. For the model of egg mass, we also tested the interaction between hormone and laying order. For Courtship and Aggression by weeks (see above), we also tested the interactions between hormone treatment and week as it represented the behavioural change over time between different treatment groups.

In all models, multi-collinearity was checked and all variance inflation factors were <1.5.

Ethical note

This study mainly involved just observations and nest checks in a very large aviary, minimizing any negative effect on the welfare of the birds. Before the observations started, however, we had to capture all pigeons to check their leg bands and measure their body mass (31 March, 2014). For this we attached a large green curtain to a rolling trail on the roof of the aviary at one end a few days earlier. On the day of capture, researchers and animal care takers (about six people) slowly approached the pigeon flock from the opposite side of the aviary. When pigeons had flown into the curtain-confined area, we closed the curtain and then caught pigeons with hand nets. The capture procedure was repeated two to three times to catch all pigeons, with breaks of a couple of hours in

between. The whole capture plus processing took about 6 hours. After processing, birds were released back to the aviary and stayed undisturbed for 10 days before the formal experiment started (10 April). This procedure never led to any death or ill health in the birds. The procedure of this study was approved by the animal welfare committee of the University of Groningen (DEC No. 5635E).

The birds were offspring generated in a series of in ovo T injection experiments. In these previous experiments, injected eggs were cross-fostered to genetically unrelated parents. All chicks were raised by their foster parents under an ad libitum food condition. We regularly monitored chick growth every 2-3 days until fledging (ca. 26 days after hatching). At various ages before fledging, a few physiological tests were conducted according to each experiment. In 2011, blood samples were taken for molecular sexing (< 150 µl, day 8-10) and hormone assay (ca. 300 µl on day 25). In 2012, only a drop of blood (ca. 15 µl) was taken on the hatching day for molecular sexing. Around day 30, a sheep red blood cell (SRBC) test was conducted to evaluate the immunocompetence of the fledglings and two blood samples were taken before and one week after SRBC injection (both <300 µl). In 2013, blood samples were taken on the hatching day (ca. 15 µl, for sexing) and day 6 (100 to ca. 150 µl, for bacterial-killing assay to assess the constitutional immunity). On day 7, we challenged chicks with lipopolysaccharides (LPS), and a small blood sample (<75 µl) was taken as a pre-LPS challenge sample. On day 8, we took another blood sample (<75 µl) to measure the titres of blood haptoglobin, an acute phase protein. Because of the serial blood sampling, we specially ensured that the total amount of blood taken was below the recommended limit of < 8ml/kg body mass within 14 days (van Zutphen et al. 1995). For our pigeons, a 1-week-old squab is about 100 g, making the limit of blood sampling 800 µl. In all years, our blood sampling was far below this limit. All these experimental procedures were approved by the Animal Welfare Committee of the University of Groningen (DEC No. 5635A, 5635D, 5635F, respectively).

In the three years of the study, chick survival before fledging was 78.79%, 95.83%, 92.31% for chicks from T-injected eggs, and 68.97%, 100%, 81.48% for chicks from oil-injected eggs. There was no statistical difference in nestling survival between two experimental groups (*t* test, $t_{3.196}$=-0.528, *P*=0.632). Post-fledging survival untill the observation period was high for both groups (T: 88.46%, 95.65%, 75% for the three years; Oil: 80%, 96%, 72.73%). There was no statistical difference in post-fledging survival either (*t* test, $t_{3.937}$=0.378, *P*=0.725).

Results

Body mass

On average, body mass was 285.15±26.85 g for T-pigeons and 284.82±26.31 g for C-pigeons (mean±SD). The difference was not statistically significant (GLM, estimate±SE= 1.597±4.319, t_{113}=0.369, *N*=118, *P*=0.713, for details see Table S4.1). Males were significantly heavier than females (estimate±SE=20.798±4.351, t_{113}=4.780, *P*<0.001), but there was no sex-specific effect of prenatal T on body mass (*P*=0.854). Age (1, 2 or 3 years) significantly predicted pigeon body mass and both the linear and quadratic terms were significant (Age, *P*=0.001; Age^2, *P*=0.002, Table S4.1). Post-hoc comparisons indicated that the first-year pigeons were significantly lighter than older ones (Tukey HSD, first year versus second year, *P*<0.001; first year versus third year, *P*=0.035), whereas there was no significant difference in body mass between the two older age groups (*P*=0.377, Fig. S4.1). The interaction between hormone treatment and age was not significant (*P*>0.19)

Nestbox occupation and pair formation

In total, 98 experimental pigeons successfully occupied a nestbox or a nest site, and the remaining 21 did not. The binomial GLM showed that prenatal T did not affect the odds of nestbox occupation (82.54% for T-pigeons and 82.14% for C-pigeons, estimate±SE= -0.01±0.486, *P*=0.984, Table S4.2), nor did the sex (*P*=0.343, Table S4.2). No interaction effects were significant (*P*>0.2).

Among the 98 pigeons that successfully occupied a nestbox or nest site, we identified 36 pairs of which both partners were from injected eggs. Among these 36 pairs, pair formation deviated significantly from a random combination by prenatal T treatment (Chi-square test, χ^2_1=4.012, *P*=0.045). More than twice as many pairs had the opposite treatment (13 T-male C-female pairs and 12 C-male T-female pairs) than the pairs having the same treatment (six C-C pairs and five T-T pairs).

Egg-laying and egg mass

From 16 April to 16 May, 2014, we recorded the laying date of the first eggs from 53 nests. Among these nests, we identified 44 males and 37 females that were born from the experimental eggs. The models showed that neither prenatal T nor parental age significantly affected the timing of egg-laying (GLM, all *P* values>0.1, Table S4.3). There was also no significant interaction between prenatal T and age (*P*>0.3). Since we found a strong deviation from random pair formation with regard to the prenatal T treatment, we

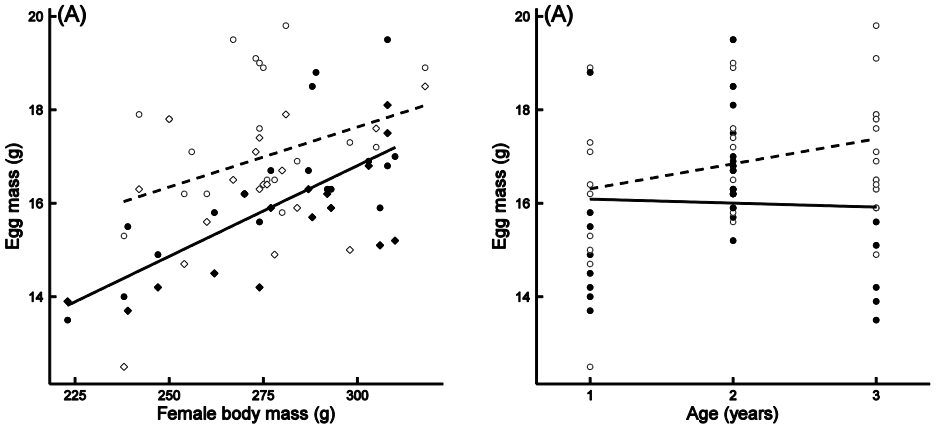

Figure 4.1 Prenatal yolk testosterone levels significantly affected females on their egg mass. (A) Egg mass plotted against female body mass. Eggs laid by T-females: filled circles and diamonds, solid line. Eggs laid by C-females: open circles and diamonds, dashed line. Circles depict the first eggs of a clutch and diamonds depict the second eggs of a clutch. (B) Egg mass plotted against female age. Eggs laid by T-females: filled dots and solid line. Eggs laid by C-females: open dots and dashed line. For simplicity, eggs of different laying order were not plotted with different symbols, but their interdependency was considered in the statistical model (Table S4.4).

examined whether the pair combination would explain the variation in egg-laying initiation with another simple linear model only including the pair combination as the predictor, but it was not significant (N=33, P=0.694).

In total, we collected 71 eggs from 37 experimental females. Prenatal T treatment significantly reduced egg mass (GLMM, estimate±SE=-1.110±0.352, t=-3.154, P=0.002, Fig. 4.1A, for model details see Table S4.4). Female body mass showed a significant positive relationship with egg mass (estimate±SE=0.034±0.007, t=4.606, P<0.001) and the second eggs of a clutch weighed significantly less than the first eggs (estimate±SE=-1.066±0.177, t=-6.008, P<0.001). There was no interaction between prenatal T treatment and laying order (P=0.173), while there was an almost significant effect of prenatal T by female age interaction (P=0.059). This marginal effect, albeit not significant, suggested that only for C-females, egg mass increased with female age, which was not apparent in T-females (Fig. 4.1B).

Table 4.3 Results of generalized linear models on pigeon behaviour.

	Estimates	SE	*t or z*	*P*
Total frequency of male courtship (N=69)				
Age	-0.061	0.047	-1.293	0.200
Hormone (T)	-0.040	0.073	-0.546	0.587
Hormone × Age	-0.054	0.096	-0.563	0.575
Total frequency of male aggression (N=69)				
Age	0.008	0.033	0.243	0.809
Hormone (T)	-0.125	0.051	-2.435	0.018
Hormone × Age	-0.055	0.067	-0.821	0.414
Total frequency of being courted in females (N=50)				
Age	1.014	0.287	3.538	0.001
Age^2	-0.225	0.071	-3.165	0.003
Hormone (T)	-0.116	0.069	-1.668	0.102
Hormone × Age	0.630	0.579	1.088	0.282
Hormone × Age^2	-0.135	0.143	-0.945	0.350
Pair-bonding behaviour (N=119)				
Age	0.153	0.246	0.623	0.533
Hormone (T)	0.107	0.384	0.278	0.781
Sex (male)	0.274	0.387	0.710	0.478
Hormone × Age	0.507	0.499	1.015	0.310
Hormone × Sex	0.247	0.781	0.316	0.752

The total frequency of behaviour was square-root-transformed to ensure the normality of the model residuals. The "Pair-bonding" was coded as 1 or 0 and the binomial error distribution with a logit link function was specified in the model. In the pair-bonding model, *z* values instead of *t* values are presented. All *P* values for main factors were derived from the model without any interaction term.

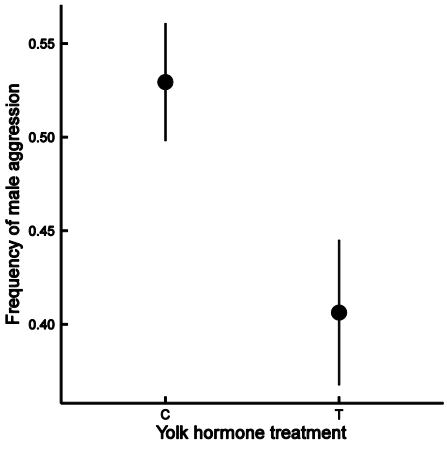

Figure 4.2 Total frequency of observed aggressive behaviour in males from testosterone-injected eggs (T) and males from control oil-injected eggs (C). The figure presents mean±SE of square-root-transformed data.

Male courtship and aggressive behaviour

The overall total frequency of male courtship behaviour was not significantly different between T-males and C-males, or among the three age groups (P=0.200 and P=0.587, respectively, Table 4.3). Prenatal T and male age did not show significant interactions on male courtship behaviour either (P=0.575). However, the total frequency of aggression in T-males was significantly lower than in C-males (P=0.018, Table 4.3, Fig. 4.2). Male age did not significantly affect male aggressive behaviour (P=0.809), and did not interact with prenatal T treatment (P=0.414, Table 4.3). We additionally analysed three subtypes of male courtship with separate models. In the separate GLMs for tail-dragging and bowing, the results showed that prenatal T, age or their interaction did not have significant effects (all P>0.1). For the behaviour driving females, we ran three Kruskal-Wallis tests for prenatal T treatment, male age, and their interactions, because the model residuals were against normality even after transformation, violating the assumption of parametric analyses. None of them showed significant effects (all P>0.5).

Both male courtship and aggression significantly increased during the 6 weeks of observation (GLMM, N=69, P<0.001, Table 4.4). For courtship behaviour, there was no overall difference between T- and C-males (P=0.465, Table 4.4) nor was there a different pattern over time (Hormone × Week, P=0.439). The interaction between hormone treatment and age was also insignificant (P=0.674).

For aggressive behaviour, we found that T-males were significantly less aggressive than C-males (P=0.004, Table 4.4). Interestingly, the significant interaction effect between hormone treatment and week (P=0.026, Table 4.4) suggested that the increase in aggression over time was less steep in T-males than in C-males (Fig. 4.3). The interaction between prenatal T and age on aggressive behaviour was not significant (P=0.488), suggesting that the overall lower aggression in T-males did not diminish across the age range of this study.

Female courtship, aggression, and being courted

Prenatal T did not significantly influence the overall frequency with which females were being courted (P=0.102, Table 4.3). Older females were courted significantly more often (P=0.001, Table 4.3). The quadratic effect of age was also significant (P=0.003, Table 4.3). Post-hoc tests further indicated that the first-year females were courted less than the 2nd and 3rd year old females (Tukey HSD, first year versus second year, P<0.001; first year versus third year, P=0.032; second year versus third year, P=0.393, Fig. S4.2). Prenatal T,

however, did not significantly interact with female age to influence the frequency of being courted (P>0.28, Table 4.3).

Compared to males, the frequency of courtship and aggression in females was low (Fig. S4.3). By re-coding the data as 1/0 and analysing with binomial GLMs, we did not find evidences suggesting higher odds of courtship and aggression in T-females than in C-females (P=0.282, 0.375, respectively, Table S4.5). We additionally explored the two female courtship behaviours, tail-dragging and nest-cooing, separately and found that T-females performed significantly more tail-dragging than C-females (P=0.015, Table S4.5) but not more nest-cooing (P=0.253).

Pair-bonding behaviour

We did not find evidence suggesting that prenatal T affected pair-bonding behaviour (P=0.781, Table 4.3). There was no significant difference in pair-bonding behaviour between age groups or between sexes, either (P=0.533 and 0.478, respectively, Table 4.3). There was no sex-specific or age-specific effect of prenatal T as the interactions were both nonsignificant (P=0.752 and 0.310, respectively, Table 4.3).

Table 4.4 Results of generalized linear mixed models on male courtship and aggression over the course of the observation period.

	Estimates	SE	*z*	*P*
Male courtship (N=69)				
Age	-0.178	0.158	-1.127	0.260
Hormone (T)	-0.178	0.244	-0.730	0.465
Week	0.528	0.074	7.131	<0.001
Hormone × Age	-0.135	0.320	-0.421	0.674
Hormone × Week	0.111	0.143	0.775	0.439
Male aggression (N=69)				
Age	-0.041	0.168	-0.241	0.809
Hormone (T)	-0.748	0.263	-2.847	0.004
Week	0.357	0.067	5.336	<0.001
Hormone × Age	-0.239	0.344	-0.694	0.488
Hormone × Week	-0.296	0.133	-2.225	0.026

Behavioural data were separated by weeks and coded as 1/0. Binomial error distribution was specified. Pigeon identity was included as a random factor. Degree of freedom is not presented as it can only be approximated in mixed models. *P* values were derived via Laplace approximation and all *P* values for main factors were derived from the model without any interaction term.

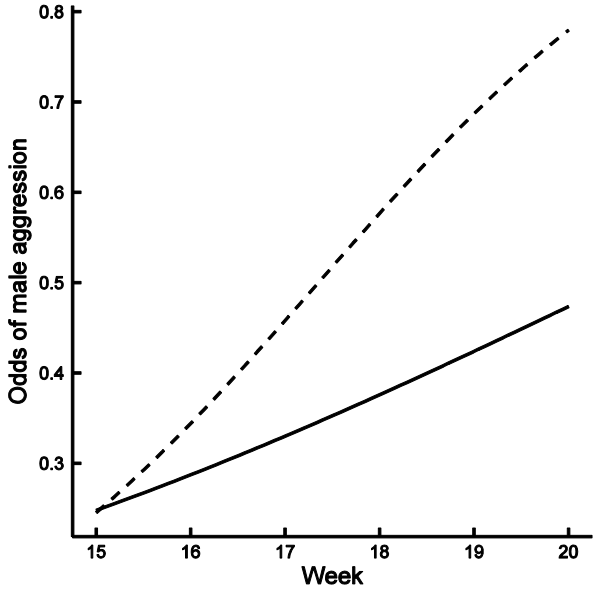

Figure 4.3 The performance of male aggressive behaviour in relation to the week of the study. For each week, the observed aggressive behaviour of each pigeon was coded as 1 (yes) or 0 (no). The plot represents fitted lines of the logistic regression for T- (solid line) and C-males (dashed line).

Discussion

Maternal effects mediated by prenatal testosterone (T) exposure have been extensively studied in birds. To evaluate its fitness consequences, the effects have to be analysed not only in the phase of development and growth, but also in adulthood. So far this has been done only until the juvenile stage or the first breeding season. This is, to our knowledge, the first study that has followed experimental birds of multiple age groups and acquired data of long-lasting effects of prenatal T exposure in birds up to their third year of life, i.e. 2 years beyond the age of first reproduction. In this study, we found that for T-males, the performance of aggressive behaviour was significantly reduced, and for T-females, egg mass was reduced. We also found that these effects of prenatal T did not wane over time while the effect on egg mass even tended to increase. In addition, we found that T injection in ovo within the physiological range caused disassortative pairing with respect to yolk T treatment of both partners.

Reduced aggression in T-males

In males, we found that the elevated concentrations of yolk T within the physiological range, mimicking in first eggs of a clutch, the naturally elevated T levels of second eggs, decreased male aggression in adulthood, not only in overall frequency (Fig. 4.2), but also the extent of the increase over time (Fig. 4.3). Several other studies have found the

opposite in juvenile or 1-year-old individuals of two other bird species (black-headed gulls, Eising et al. 2006; house sparrows, Strasser and Schwabl 2004; Partecke and Schwabl 2008), and no effect in chickens (Riedstra et al. 2013). This suggests species-specific differences in the behavioural response to yolk T, as has been found for hatching time (von Engelhardt and Groothuis 2011). Alternatively, the effects may be context dependent. However, the studies on the gulls and house sparrows were conducted, like ours, in semi-natural conditions using dosages within the physiological range of the species, suggesting species-specific effects. These effects might not come about because yolk T has modified endogenous T production in adulthood, since many studies have reported no effects of prenatal T exposure on plasma T levels (Riedstra et al. 2013; Schweitzer et al. 2013; Rubolini et al. 2014), including some studies that found increased aggressive behaviour (e.g. Partecke and Schwabl 2008). Alternatively, the elevated prenatal T exposure may have affected the sensitivity to this hormone. Support for this comes, albeit in 2-week-old domestic chicks, from one study showing decreased mRNA expression levels of androgen receptors in the hypothalamic regions of the brain as a consequence of elevated yolk T concentrations (Pfannkuche et al. 2011).

Whether the changed behavioural phenotype will lead to fitness differences is as yet unclear. In our results, although males from T-injected eggs were less aggressive, this seemed not to decrease their chance of occupying nestboxes or breeding, as we found no difference in nestbox occupation and egg-laying initiation. Nevertheless, the costs of being less aggressive may still be manifest in the wild where competition over nest-sites, females and food may be more severe than in our aviary. Although they did not measure aggressive behaviour, two previous studies in collared flycatchers (*Ficedula albicollis*) both showed that males exposed to higher prenatal T had lower fitness return (Ruuskanen et al. 2012; Tschirren et al. 2014).

Reduced egg mass laid by T-females

For females, the long-term effects of prenatal T appeared in egg mass. This may be disadvantageous to the fitness of females and their mates as lower egg mass is known to have detrimental effects on the offspring (Krist 2011). Perhaps elevated exposure to prenatal T interferes with the egg-producing physiology. A previous study in Chinese quails (*Coturnix chinensis*) also reported reduced egg mass for females exposed to higher yolk T (Uller et al. 2005). In ring-necked pheasants (*Phasianus colchius*), elevated prenatal T exposure also negatively affected the egg-laying of females and reduced the proportion of fertilized eggs, although egg mass itself was not significantly affected (Rubolini et al. 2007). However, there is also one study with opposite results: female canaries from T-injected

eggs more often laid a larger clutch than the control females, and their egg mass was not affected (W. Müller et al. 2009). This species difference is consistent with its effect in the chick phase in canaries that yolk T also stimulated competitive begging behaviour for food (Schwabl 1996), whereas in poultry, yolk T suppressed behavioural competition for food (Riedstra et al. 2013). Since egg mass is strongly related to offspring quality (Krist 2011) and is also partly determined by maternal body mass (Fig. 4.1A), our results suggest a likely non-genetic pathway of trans-generational effects by prenatal exposure to maternal hormones. The hormone levels deposited into the eggs by the parental generation may shape the phenotypes of the F1 generation by producing smaller offspring from smaller eggs, which in turn may lead to smaller eggs in the next generation (Burton and Metcalfe 2014). Additionally, if the yolk hormone content is also affected by the prenatal hormone exposure in the previous generation, it depicts a non-genetic inheritance pathway across generations in parallel with the genetically inheritable variation in both egg mass and yolk hormone deposition (Tschirren et al. 2009; Okuliarová et al. 2011; W. Müller et al. 2012). Such a potential combination of direct and indirect genetic effects may considerably affect the rate of evolution (Moore et al. 1998).

Interestingly, our results did not show any evidence that the long-term effects of prenatal T would diminish in older birds. The only significant interaction between prenatal T and age even indicates that the effect of T on egg mass became stronger in older females (Fig. 4.1B), suggesting that maternal T might affect the rate of senescence. Indeed, several studies indicate a decline in egg quality after a certain age as a result of maternal ageing (Beamonte-Barrientos et al. 2010; Preston et al. 2014). Perhaps elevated exposure to prenatal T incurs higher costs in older females, for example by influencing the state of oxidative balance (Tobler and Sandell 2009; Galván and Alonso-Alvarez 2010; Noguera et al. 2011; Tobler et al. 2013) and the accumulation of oxidative damage. Further studies are certainly needed to test this hypothesis.

Non-random pair-bonding based on prenatal T treatment

Rock pigeons tend to maintain a life-time pair-bond unless their partner dies, and both sexes participate in incubating and parental care (Johnston and Janiga 1995). Mate choice in this species could therefore have very important fitness effects. Interestingly, we found a significant non-random pattern of pair formation with regard to the prenatal T treatment: many more T-C and C-T pairs than T-T and C-C pairs. This finding is consistent with the study in ring-necked pheasants, in which there were significantly more copulations between T-males and C-females (Bonisoli-Alquati et al. 2011). In rock pigeons, female mate choice is probably more important than male mate choice, because males

frequently court many females and females typically lack courtship behaviour. A likely scenario according to our results would then be that compared to the same-sex counterparts, C-females are dominant over T-females (in line with yolk T suppressing aggression of males) and prefer T-males, leaving T-females to pair with C-males. An alternative scenario could be that T-males are dominant over C-males and they prefer to pair with C-females because of better reproductive capacity (laying larger eggs). In this scenario, the lower levels of aggression in T-males could reflect their earlier pair-bonding. However, this seems not supported by our result that T-males did not have their first clutches of eggs earlier than C-males. In either scenario, why T-males, being less aggressive, might be more attractive or dominant, and show no difference in courtship behaviour relative to controls, is unclear. Perhaps yolk T has influenced their plumage colouration, as the iridescent coloration on the neck is probably sexually selected as the display of the throat plumage is a common feature of courtship in this genus (Baptista et al. 1997). A positive effect of yolk T on nuptial plumage in black-headed gulls and the black bib size in male house sparrows has been found earlier (Strasser and Schwabl 2004; Eising et al., 2006).

No effect on sexual differentiation

We never saw females of either treatment performing male-like courtship behaviour or aggression, nor males showing female behaviour. Intriguingly, our results indicated higher odds of observed tail-dragging behaviour in T-females than in C-females (Table S4.5). However, since this behaviour is normal female courtship, it does not suggest any signs of masculinization. Second, during our observations, the copulation position of females never deviated from the normal pattern. This indicates that prenatal exposure to T within the normal range that females deposit in their eggs does not affect the process of sexual differentiation in females. The decreased frequency of aggression in T-males may indicate a slight effect of de-masculinization in males, as has been suggested for chicks of laying hens (Riedstra et al. 2013). This assumes that, as males are the default sex in birds and oestradiol is the feminizing hormone, T is aromatised in the brain to oestradiol. However, the effect is by far much subtler than that in the classical experiments with gonadal hormones on sexual differentiation in birds (for a review, see e.g. Balthazart et al. 2009). These experiments, however, used extremely high dosages that may not reflect the natural process (Carere and Balthazart 2007; Groothuis and Schwabl 2008). Studies on the effect of gonadal hormone manipulations within the physiological range should therefore receive more attention also in the field of sexual differentiation.

Conclusions

In conclusion, we found long-lasting effects of prenatal T exposure on reproductive behaviour (male aggression and female tail-dragging), pair formation and egg mass even when birds had reached 3 years of age. This indicates that maternal effects may reach far into adulthood, and should be taken into consideration when assessing fitness effects. This also opens research lines into the underlying physiological mechanisms.

Whether prenatal T exposure actually has life-long impact, even on the process of ageing, requires really long-term data to verify. Its potential fitness effects need tracking studies on birds from yolk T manipulation studies in the field. Our results should hopefully stimulate physiological and neurobiological studies on the long term effects of early exposure to testosterone both in and outside the context of sexual differentiation.

Acknowledgements

We thank the anonymous reviewers who greatly helped us improve the manuscript. We also thank all colleagues and animal care takers for invaluable support during the practical work, especially for capturing and handling pigeons. The help of the following students during the pilot stages and the formal observations of this study is greatly appreciated: Thijmen Piek, Yvonne Oldenhof, Christoph Gahr, Frederieke Vlieg, Marjet Horst, Lisette van Steenis, Evelyne Witte, Bernice van den Berg, Reinoud Brenkman, Irene van Dijken, and Renate Kat. B.Y.H. was financially supported by the scholarship of overseas study from the Ministry of Education, Taiwan, and by a grant to T.G.G.G from the University of Groningen.

References

Balthazart, J., Arnold, A.P., Adkins-Regan, E., 2009. Sexual differentiation of brain and behaviour in birds. In Pfaff, D.W., Arnold, A.P., Fahrbach, S.E., Etgen, A.M., Rubin, R.T. (Eds.), *Hormones, Brain and Behavior*, pp. 1745-1789. Elsevier, Amsterdam, The Netherlands.

Baptista, L.F., Trail, P.W., Horblit, H.M., 1997. Family Columbidae. In del Hoyo, J., Elliott, A., Sargatal, J. (Eds.), *Handbook of the Birds of the World, Vol. 4 -- Sandgrouse to Cuckoos*, pp. 60-245. Lynx Edicions, Barcelona, Spain.

Bates, D., Maechler, M., Bolker, B., Walker, S., 2014. lme4: Linear mixed-effects models using Eigen and S4. R package version 1.1-7. Retrieved from http://CRAN.R-project.org/package=lme4.

Beamonte-Barrientos, R., Velando, A., Drummond, H., Torres, R., 2010. Senescence of maternal effects: aging influences egg quality and rearing capacities of a long-lived bird. *The American Naturalist* 175: 469-480.

Bonisoli-Alquati, A., Matteo, A., Ambrosini, R., Rubolini, D., Romano, M., Caprioli, M., Dessì-Fulgheri, F., Baratti, M., Saino, N., 2011. Effects of egg testosterone on female mate choice and male sexual behavior in the pheasant. *Hormones and Behavior, 59*, 75-82.

Bonisoli-Alquati, A., Rubolini, D., Caprioli, M., Ambrosini, R., Romano, M., and Saino, N. (2011) Egg testosterone affects wattle color and trait covariation in the ring-necked pheasant. *Behavioral Ecology and Sociobiology* 65: 1779-1790.

Burton, T., Metcalfe, N.B., 2014. Can environmental conditions experience in early life influence future generations? *Proceedings of the Royal Society B: Biological Sciences* 281: 20140311.

Carere, C., Balthazart, J., 2007. Sexual versus individual differentiation: the controversial role of avian maternal hormones. *Trends in Endocrinology and Metabolism,* 18: 73-80.

Eising, C.M., Müller, W., Groothuis, T.G.G., 2006. Avian mothers create different phenotypes by hormone deposition in their eggs. *Biology Letters* 2: 20-22.

Galván, I., Alonso-Alvarez, C., 2010. Yolk testosterone shapes the expression of a melanin-based signal in great tits: an antioxidant-mediated mechanism? *The Journal of Experimental Biology* 213: 3127-3130.

Gil, D., 2008. Hormones in avian eggs: physiology, ecology and behavior. *Advances in the Study of Behavior* 38: 337-398.

Gilbert, S.F., 2001. Ecological developmental biology: developmental biology meets the real world. *Developmental Biology* 233: 1-12.

Groothuis, T.G.G, Schwabl, H., 2008. Hormone-mediated maternal effects in birds: mechanisms matter but what do we know of them? Philosophical Transactions of the Royal Society B: 363: 1647-1661.

Groothuis, T.G.G., Müller, W., von Engelhardt, N., Carere, C., Eising, C., 2005. Maternal hormones as a tool to adjust offspring phenotype in avian species. *Neuroscience and Biobehavioral Reviews* 29: 329-352.

Hetmański, T., Barkowska, M., 2007. Density and age of breeding pairs influence feral pigeon, *Columba livia* reproduction. *Folia Zoologica* 56: 71-83.

Hsu, B.-Y., Dijkstra, C., Darras, V.M., de Vries, B., Groothuis, T.G.G., 2016. Maternal adjustment or constraint: differential effects of food availability on maternal deposition of macro-nutrients, steroids and thyroid hormones in rock pigeon eggs. *Ecology and Evolution* 6: 397-411.

Johnston, R.E., Janiga, J., 1995. *Feral Pigeons*. Oxford University Press, New York, New York.

Krist, M., 2011. Egg size and offspring quality: a meta-analysis in birds. *Biological Reviews* 86: 692-716.

Moore, A.J., Wolf, J.B., and Brodie III, E.D., 1998. The influence of direct and indirect genetic effects on the evolution of behaviour: social and sexual selection meet maternal effects. In Mousseau, T.A., Fox, C.W. (Eds.), *Maternal Effects as Adaptations*, pp. 22-41. Oxford University Press, New York, New York.

Mousseau, T.A., Fox, C.W., 1998. *Maternal Effects as Adaptations*. Oxford University Press, New York, New York.

Müller, M.S., 2013. *Proximate and ultimate aspects of androgen-mediated maternal effects in relation to sibling competition in birds*. PhD dissertation. University of Groningen, Groningen, The Netherlands.

Müller, W., Eens, M., 2009. Elevated yolk androgen levels and the expression of multiple sexually selected male characters. *Hormones and Behavior* 55: 175-181.

Müller, W., Lessells, C.M., Korsten, P., von Engelhardt, N., 2007. Manipulative signals in family conflict? On the function of maternal yolk hormones in birds. *The American Naturalist,* 169: E84-E96.

Müller, W., Vergauwen, J., Eens, M., 2008. Yolk testosterone, postnatal growth and song in male canaries. *Hormones and Behavior* 54: 125-133.

Müller, W., Vergauwen, J., Eens, M., 2009. Long-lasting consequences of elevated yolk testosterone levels on female reproduction. *Behavioral Ecology and Sociobiology* 63: 809-816.

Müller, W., Goerlich, V.C., Vergauwen, J., Groothuis, T.G.G., Eens, M., 2012. Sources of variation in yolk hormone deposition: consistency, inheritance and developmental effects. *General and Comparative Endocrinology* 175: 337-343.

Noguera, J.C., Alonso-Alvarez, C., Kim, S.-Y., Morales, J., Velando, A., 2011. Yolk testosterone reduces oxidative damages during postnatal development. *Biology Letters* 7: 93-95.

Okuliarová, M., Groothuis, T.G.G., Škrobánek, P., Zeman, M., 2011. Experimental evidence for genetic heritability of maternal hormone transfer to offspring. *The American Naturalist* 177: 824-834.

Partecke, J., Schwabl, H., 2008. Organizational effects of maternal testosterone on reproductive behavior of adult house sparrows. *Developmental Neurobiology* 68: 1538-1548.

Pfannkuche, K.A., Gahr, M., Weites, I.W., Riedstra, B., Wolf, C., Groothuis, T.G.G., 2011. Examining a pathway for hormone mediated maternal effects – yolk testosterone affects androgen receptor expression and endogenous testosterone production in young chicks (*Gallus gallus domesticus*). *General and Comparative Endocrinology* 172: 487-493.

Preston, B.T., Jalme, M.S., Hingrat, Y., Lacroix, F., Sorci, G., 2014. The sperm of aging male bustards retards their offspring's development. *Nature Communications* 6: 6146.

R core team, 2013. R: A language and environment for statistical computing. R Foundation for statistical Computing, Vienna, Austria. Retrieved from http://www.R-project.org/.

Riedstra, B., Pfannkuche, K.A., Groothuis, T.G.G., 2013. Increased exposure to yolk testosterone has feminizing effects in chickens, *Gallus gallus domesticus*. *Animal Behaviour* 85: 701-708.

Rubolini, D., Romano, M., Martinelli, R., Leoni, B., and Saino, N., 2006. Effects of prenatal yolk androgens on armaments and ornaments of the ring-necked pheasant. *Behavioral Ecology and Sociobiology* 59: 549-560.

Rubolini, D., Martinelli, R., von Engelhardt, N., Romano, M., Groothuis, T. G. G., Fasola, M., Saino, N., 2007. Consequences of prenatal androgen exposure for the reproductive performance of female pheasants (*Phasianus colchicus*). *Proceedings of the Royal Society B: Biological Sciences* 274: 137-142.

Rubolini, D., Fusani, L., Bonisoli-Alquati, A., Canoine, V., Caprioli, M., Romano, M., Ambrosini, R., Dessì-Fulgheri, F., Saino, N., 2014. Effects of egg and circulating testosterone on ring-necked pheasant (*Phasianus colchicus*) male traits and combat outcome. *Ethology* 120: 64-76.

Ruuskanen, S., Laaksonen, T., 2010. Yolk hormones have sex-specific long-term effects on behavior in the pied flycatcher (*Ficedula hypoleuca*). *Hormones and Behavior* 57: 119-127.

Ruuskanen, S., Doligez, B., Pitala, N., Gustafsson, L., Laaksonen, T., 2012. Long-term fitness consequences of high yolk androgen levels: sons pay the costs. *Functional Ecology* 26: 884-894.

Schwabl, H., 1996. Maternal testosterone in the avian egg enhances postnatal growth. *Comparative Biochemistry and Physiology* 114A: 271-276.

Schwabl, H., Holmes, D., Strasser, R., Scheuerlein, A., 2012. Embryonic exposure to maternal testosterone influences age-specific mortality patterns in a captive passerine bird. *Age* 34: 87-94.

Schweitzer, C., Goldstein, M.H., Place, N.J., Adkins-Regan, E., 2013. Long-lasting and sex-specific consequences of elevated egg yolk testosterone for social behavior in Japanese quail. *Hormones and Behavior* 63: 80-87.

Strasser, R., Schwabl, H., 2004. Yolk testosterone organizes behavior and male plumage coloration in house sparrows (*Passer domesticus*). *Behavioural Ecology and Sociobiology* 56: 491-497.

Tobler, M., Sandell, M.I., 2009. Sex-specific effects of prenatal testosterone on nestling plasma antioxidant capacity in the zebra finch. *The Journal of Experimental Biology* 212: 89-94.

Tobler, M., Sandell, M.I., Chiriac, S., Hasselquist, D., 2013. Effects of prenatal testosterone exposure on antioxidant status and bill color in adult zebra finches. *Physiological and Biochemical Zoology* 86: 333-345.

Tschirren, B., Fitze, P.S., Richner, H., 2007. Maternal modulation of natal dispersal in a passerine bird: an adaptive strategy to cope with parasitism? *The American Naturalist* 169: 87-93.

Tschirren, B., Sendecka, J., Groothuis, T.G.G., Gustafsson, L., Doligez, B., 2009. Heritable variation in maternal yolk hormone transfer in a wild bird population. *The American Naturalist* 174: 557-564.

Tschirren, B., Postma, E., Gustafsson, L., Groothuis, T.G.G., Doligez B., 2014. Natural selection acts in opposite ways on correlated hormonal mediators of prenatal maternal effects in a wild bird population. *Ecology Letters* 17: 1310-1315.

Uller, T., Eklöf, J., Andersson, S., 2005. Female egg investment in relation to male sexual traits and the potential for transgenerational effects in sexual selection. *Behavioral Ecology and Sociobiology* 57: 584-590.

Van Zutphen, L.F.M., Baumans, V., Beynen, A.C., 1995. *Proefdieren en dierproeven*. Wetenschappelijke uitgeverij Bunge, Utrecht, The Netherlands (In Dutch).

Vergauwen, J., Eens, M., Müller, W., 2014. Consequences of experimentally elevated yolk testosterone levels for intra- and inter-sexual selection in canaries. *Behavioral Ecology and Sociobiology* 68: 1299-1309.

von Engelhardt, N., Groothuis, T.G.G., 2011. Maternal hormones in avian eggs. In Norris, D.O., Lopez, K.H. (Eds.), *Hormones and Reproduction of Vertebrates, Vol. 4 – Birds*, pp.91-127. Elsevier, Amsterdam, The Netherlands.

Supplementary materials

Table S4.1 Results of general linear model on pigeon body mass.

	Estimates	SE	t_{113}	*P*
Age	60.905	18.061	3.372	0.001
Age^2	-13.740	4.437	-3.097	0.002
Hormone (T)	1.597	4.319	0.369	0.713
Sex (male)	20.798	4.351	4.780	<0.001
Hormone × Age	42.953	36.473	1.178	0.241
Hormone × $(Age)^2$	-11.675	8.983	-1.300	0.196
Hormone × Sex	-1.635	8.864	-0.184	0.854

All *P* values for main factors were derived from the model without any interaction term (*N*=118, one missing value in body mass was removed).

Table S4.2 Results of generalized linear model on the odds of successfully occupying a nestbox or a nest-site.

	Estimates	SE	z_{115}	*P*
Age	0.296	0.313	0.948	0.343
Hormone (T)	-0.010	0.486	-0.020	0.984
Sex (male)	-0.483	0.510	-0.948	0.343
Hormone × Age	-0.297	0.648	-0.457	0.647
Hormone × Sex	1.297	1.086	1.194	0.232

For each individual, if it successfully occupied a nestbox or a nest-site, it was coded as 1, otherwise 0. Binomial error distribution was therefore specified. All *P* values for main factors were derived from the model without any interaction term (*N*=119).

Table S4.3 Results of general linear model on the laying date of the first eggs of the first clutches.

	Estimates	SE	*t*	*P*
Male model (N=44)				
Age	-2.276	3.047	-0.747	0.459
Hormone (T)	1.251	4.833	0.259	0.797
Hormone × Age	3.082	6.243	0.494	0.624
Female model (N=37)				
Age	-5.427	3.534	-1.536	0.134
Hormone (T)	-3.292	5.323	-0.618	0.540
Hormone × Age	7.627	7.258	1.051	0.301

Males and females were analysed with separate models. The egg-laying dates were rank-transformed to ensure normally-distributed model residuals. All *P* values for main factors were derived from the model without any interaction term.

Table S4.4 Results of general linear mixed model on egg mass.

	Estimates	SE	*t*	LRT *P*	PB *P*
Age	0.178	0.236	0.756	0.422	0.458
Hormone (T)	-1.110	0.352	-3.154	0.002	0.002
Body mass	0.034	0.007	4.606	<0.001	<0.001
Laying order (2^{nd})	-1.066	0.177	-6.008	<0.001	<0.001
Hormone × Age	-0.938	0.466	-2.013	0.036	0.059
Hormone × Laying order	0.486	0.351	1.385	0.159	0.173

Only females were included in this analysis. The nest identity was included as a random factor. In mixed models, the degree of freedom is not presented as it can only be approximated and the *P* values were derived using two approaches of model comparison: log-likelihood ratio test (LRT) and parametric bootstrapping (PB) model comparison. This is because the model residuals showed a moderate positive correlation with the fitted values. In most cases, PB model comparisons gave highly consistent results and similar to those of LRT, but for the interaction between hormone treatment and age, the *P* value was just over 0.05. We thus provide both sets of *P* values for better evaluation of the effects. All *P* values for main factors were derived from the model without any interaction term.

Table S4.5 Results of generalized linear mixed models on female courtship and aggression (N=50).

	Estimates	SE	*z*	*P*
Total courtship				
Age	-0.167	0.366	-0.456	0.649
Hormone (T)	0.620	0.577	1.075	0.282
Hormone × Age	-0.095	0.732	-0.130	0.897
Tail-dragging				
Age	-0.022	0.405	-0.055	0.956
Hormone (T)	1.630	0.673	2.422	0.015
Hormone × Age	-1.459	0.957	-1.525	0.127
Nest-cooing				
Age	-0.037	0.366	-0.102	0.918
Hormone (T)	0.661	0.578	1.143	0.253
Hormone × Age	0.138	0.733	0.189	0.850
Total aggression				
Age	-0.646	0.480	-1.345	0.179
Hormone (T)	0.653	0.735	0.888	0.375
Hormone × Age	-1.025	1.041	-0.985	0.325

All data were zero-inflated and thus re-coded as 1/0. Binomial error distribution with a logit link function was specified in the models. Degree of freedom is not presented as it can only be approximated in mixed models. *P* values were derived via Laplace approximation and all *P* values for main effects were derived from the model without any interaction term.

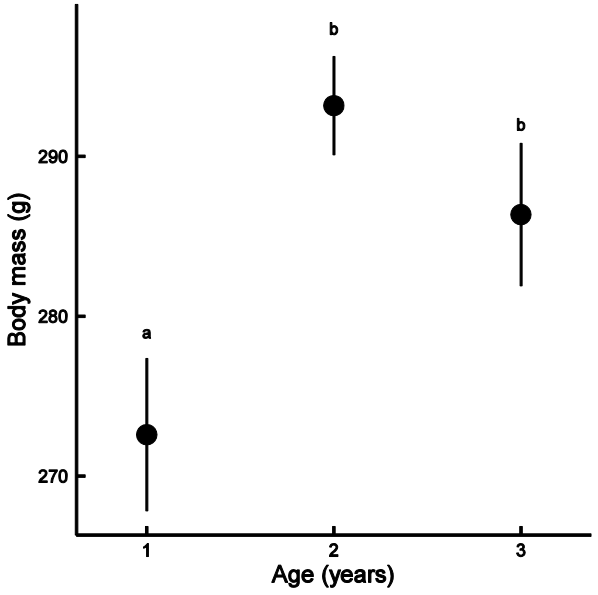

Figure S4.1 Pigeon body mass across age groups (mean±SE). Different letters represent significant difference from Tukey HSD post-hoc comparisons.

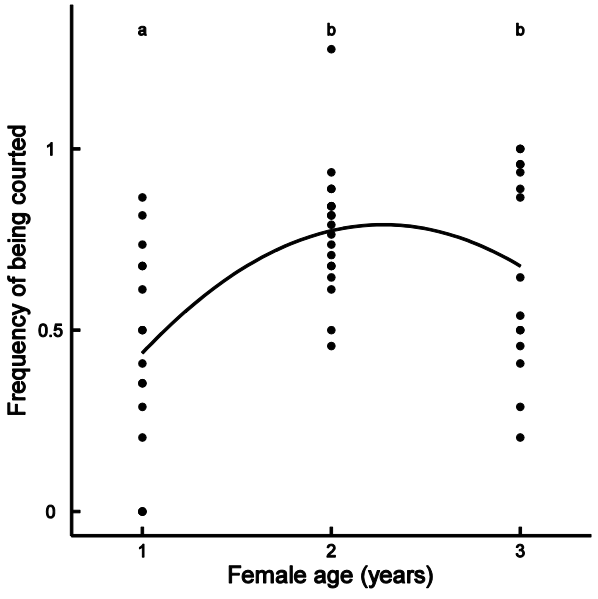

Figure S4.2 The frequency of being courted in female pigeons plotted against female age. Different letters represent significant difference from Tukey HSD post-hoc comparisons.

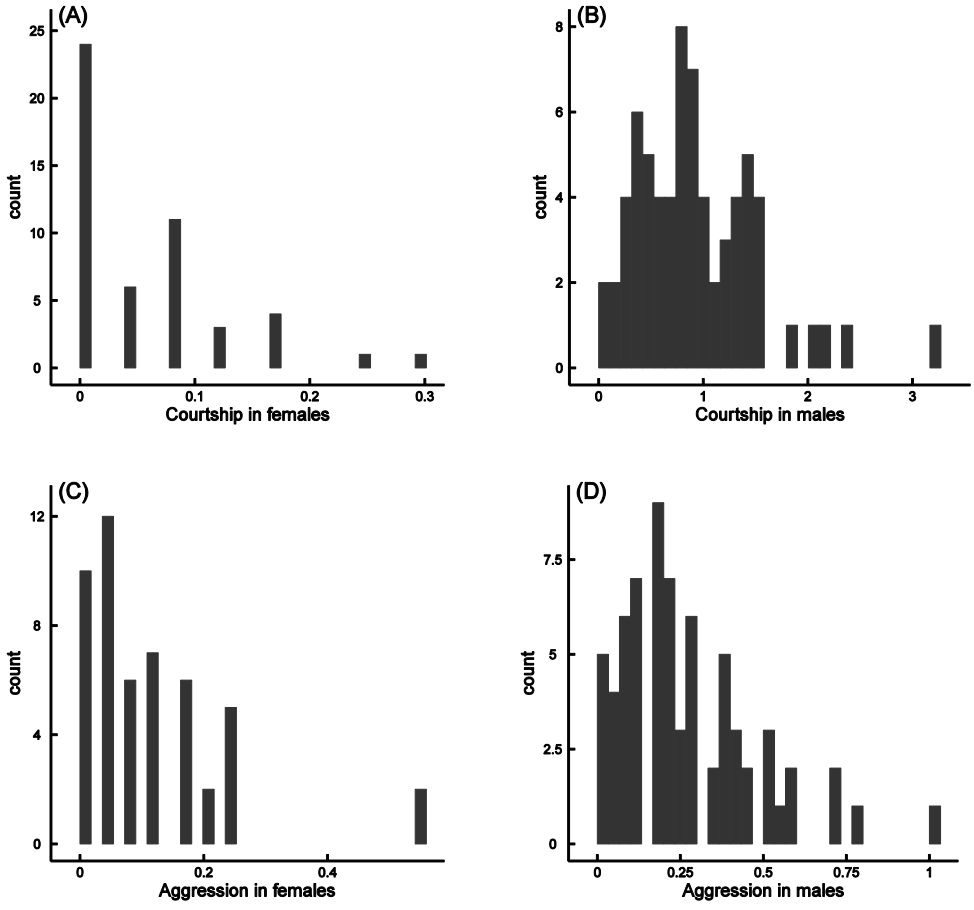

Figure S4.3 Histograms of behaviours observed in pigeons: courtship (A) courtship in females; (B) courtship in males; (C) aggression in females; (D) aggression in males